LEFT-RIGHT ASYMMETRY DETERMINATION IN VERTEBRATES

Mark Mercola
Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115; e-mail: mmercola@hms.harvard.edu

Michael Levin
Forsyth Institute, 140 The Fenway, Boston, Massachusetts 02115; e-mail: mlevin@forsyth.org

Key Words laterality, TGFβ, Nodal, Shh, cilia, gap junctions, mice, chick, Xenopus

Abstract A distinctive and essential feature of the vertebrate body is a pronounced left-right asymmetry of internal organs and the central nervous system. Remarkably, the direction of left-right asymmetry is consistent among all normal individuals in a species and, for many organs, is also conserved across species, despite the normal health of individuals with mirror-image anatomy. The mechanisms that determine stereotypic left-right asymmetry have fascinated biologists for over a century. Only recently, however, has our understanding of the left-right patterning been pushed forward by links to specific genes and proteins. Here we examine the molecular biology of the three principal steps in left-right determination: breaking bilateral symmetry, propagation and reinforcement of pattern, and the translation of pattern into asymmetric organ morphogenesis.

CONTENTS

INTRODUCTION ..................................................... 780
EARLY STEPS: ORIENTATION OF LEFT-RIGHT
ASYMMETRY WITH RESPECT TO DORSOVENTRAL
AND ANTEROPOSTERIOR BODY AXES ............................. 781
Theoretical and Practical Concerns ................................. 781
Nodal Monocilia Model ............................................. 784
Gap Junctional Communication Requirement in Chicks and Xenopus 786
Adhesion Junction Requirements .................................. 787
Left-Right Coordinator Model ..................................... 788
INTERMEDIATE STEPS: PROPAGATION AND REINFORCEMENT
OF LEFT-RIGHT POLARITY ........................................ 788
Asymmetric Cascades of Gene Expression ......................... 788
TGFβ Family Members .............................................. 789

1081-0706/01/1115-0779$14.00 779
INTRODUCTION

The development of embryos in three-dimensional space requires specification of pattern along three orthogonal axes during embryonic morphogenesis. The external body-plan of vertebrates possesses bilateral symmetry, and much research has addressed the molecular mechanisms of patterning along the anterior-posterior (AP), dorso-ventral (DV), and mediolateral body axes. However, vertebrate embryos (and indeed, many invertebrates) also exhibit a strikingly conserved left-right (LR) asymmetry of the internal organs. Nearly all visceral organs of the thorax and abdomen are LR asymmetrical in their anatomy, placement and, in some cases, physiology. LR asymmetry is conserved throughout chordate evolution, although details of anatomical asymmetry such as sidedness of the venous system can vary between species. Nonetheless, it is striking that all normal individuals of a given species have identical asymmetry (Neville 1976). In humans, for instance, complete mirror-image reversal of internal organs (situs inversus) is rare, presenting in only about 1 individual per 20,000. In some species, in particular among higher mammals, stereotypic LR asymmetry extends to the brain and nervous system, a situation that has profound implications for understanding behavior and cognition (Harnad 1977), not only of the individual, but perhaps of the species as well.

Abnormalities in the proper development of laterality often lead to discordant LR asymmetry of the internal organs and form a class of human birth defects with significant implications for the health of the individual (reviewed in Casey & Hackett 2000). Such malformations can be caused by chemical agents and heritable genetic lesions. These defects occur in more than 1 in 8000 live births and can be classified into several types. Of these, situs inversus is the only class with no major effects on the health of the individual and as such is likely to be under-detected. In contrast, isomerism (resulting in a symmetrical organ situs, e.g., asplenia or polysplenia), single organ inversions (such as dextrocardia), or heterotaxia (a loss of concordance such that some organs develop with reversed, but organotypic, asymmetry) often have grave consequences. For example, isolated dextrocardia with the abdominal viscera in the normal position is associated with various combinations of severe cardiac malformations, among the most
common being ventricular septal defects, single ventricle, valvular abnormalities and abnormalities of the systemic and pulmonary blood flow.

The establishment of left-right asymmetry is a fascinating problem in embryonic morphogenesis, and glimpses into the molecular mechanisms that determine LR asymmetry have been possible only recently. (Tables 1–3 summarize some genes and proteins involved in LR determination.) Important questions abound. Why is LR asymmetry uniform throughout a population; in other words, why don’t individuals with situs solitus co-exist in roughly equal numbers with those with situs inversus? To what extent is the structure and physiology of complex asymmetric organs such as the heart shaped directly by the genetics of LR patterning or, indirectly, by other forces such as hemodynamic influences? When did LR asymmetry appear during evolution? Did it appear only once? And is it connected to chirality in lower forms (such as direction of snail shell coiling)? It is difficult to address these questions thoroughly without a detailed understanding, at the molecular, genetic, and biochemical levels, of the formation of biased asymmetry in embryos. Thus this review summarizes recent efforts at understanding the determination of LR asymmetry in embryos. Of particular interest is the developmental stage during which LR asymmetry is initiated in vertebrates and whether diverse species orient LR asymmetry similarly.

EARLY STEPS: ORIENTATION OF LEFT-RIGHT ASYMMETRY WITH RESPECT TO DORSOVENTRAL AND ANTEROPOSTERIOR BODY AXES

Theoretical and Practical Concerns

Conceptually, LR patterning can be divided into three phases. Only in the final phase do morphological changes in cell migration, shape, proliferation, and survival combine to render asymmetric anatomy. Upstream of morphogenesis lie pathways of genes that are expressed by cell fields on only one side of the embryo’s midline. These genetic cascades form the middle phase of LR patterning and propagate information to the developing organ primordia. Whichever asymmetrically expressed gene turns out to be at the top of the emerging pathways, it is necessary to ask what acts to determine its asymmetry. Thus some mechanism must break the symmetry of the embryo and orient the LR axis with respect to the other two axes.

A conceptually satisfying model for the initial orientation of LR asymmetry was proposed by Brown & Wolpert (1990) and has influenced much of the thinking in the field. They postulated that a chiral protein, termed the F-molecule, is tethered with respect to the AP and DV axes (Figure 1). Stripped of embryological and cellular details, the model predicts that the intrinsic chirality of the F-molecule would translate AP and DV information into LR polarity. Intrinsic chirality would be coupled to cellular processes through the association of the F-molecule with proteins involved in cytoskeleton, cell division, transport,
TABLE 1  Asymmetrically expressed genes known to be involved in LR determination pathways. This and the following tables are not exhaustive; for instance, a zebrafish screen for laterality effects has uncovered numerous genes not listed here (see Chen et al. 2001)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Product/Role</th>
<th>Side</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activin-βB</td>
<td>Chick</td>
<td>TGFβ-family signaling molecule</td>
<td>Right</td>
<td>Levin et al. 1997</td>
</tr>
<tr>
<td>BMP-4</td>
<td>Zebrafish</td>
<td>BMP family signaling molecule</td>
<td>Left (heart)</td>
<td>J-N Chen et al. 1997</td>
</tr>
<tr>
<td>Act-RHa</td>
<td>Chick</td>
<td>Activin receptor</td>
<td>Right</td>
<td>Levin et al. 1995</td>
</tr>
<tr>
<td>Caronte</td>
<td>Chick</td>
<td>Cerberus/DAN family member</td>
<td>Left</td>
<td>Rodriguez Esteban et al. 1999;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yokouchi et al. 1999; Zhu et al. 1999</td>
</tr>
<tr>
<td>ePTC</td>
<td>Chick</td>
<td>Receptor</td>
<td>Left</td>
<td>Pagan-Westphal &amp; Tabin 1998</td>
</tr>
<tr>
<td>dBHAND</td>
<td>Chick, mouse</td>
<td>BHLH transcription factor</td>
<td>Right</td>
<td>Srivastava et al. 1995</td>
</tr>
<tr>
<td>eHAND</td>
<td>Chick, mouse</td>
<td>BHLH transcription factor</td>
<td>Left</td>
<td>Cserjesi et al. 1995</td>
</tr>
<tr>
<td>FGF8</td>
<td>Chick</td>
<td>Growth factor</td>
<td>Right (mouse)</td>
<td>Boettger et al. 1999, Meyers &amp; Martin 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Left (chick)</td>
<td></td>
</tr>
<tr>
<td>Fibrillin-2,</td>
<td>Chick</td>
<td>Extracellular matrix component</td>
<td>Right side</td>
<td>Smith et al. 1997</td>
</tr>
<tr>
<td>JB3 epitope</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flectinα</td>
<td>Chick</td>
<td>Extracellular matrix component</td>
<td>Left</td>
<td>Tsuda et al. 1996</td>
</tr>
<tr>
<td>Follistatin</td>
<td>Chick</td>
<td>Signaling molecule</td>
<td>Right</td>
<td>Levin 1998</td>
</tr>
<tr>
<td>HNF3-β</td>
<td>Chick</td>
<td>Winged-helix transcription</td>
<td>Left</td>
<td>Levin 1998, Levin et al. 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(antivin in</td>
<td>chick, Xenopus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xenopus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Chick</td>
<td>Adhesion molecule</td>
<td>Right node,</td>
<td>Garcia-Castro et al. 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Left groove</td>
<td></td>
</tr>
<tr>
<td>NKX1.2</td>
<td>Chick, mouse</td>
<td>Transcription factor</td>
<td>Right (mice),</td>
<td>Schneider et al. 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Left (chick)</td>
<td></td>
</tr>
<tr>
<td>Nodal</td>
<td>Chick, mouse</td>
<td>TGFβ family member</td>
<td>Left</td>
<td>Levin et al. 1995, Lowe et al. 1996; see text for additional references</td>
</tr>
<tr>
<td></td>
<td>Xenopus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shh</td>
<td>Chick</td>
<td>Signaling molecule</td>
<td>Left</td>
<td>Levin et al. 1995</td>
</tr>
<tr>
<td>SnR</td>
<td>Chick</td>
<td>Zinc finger protein</td>
<td>Right</td>
<td>Isaac et al. 1997, Selton et al. 1998</td>
</tr>
</tbody>
</table>

*Protein epitope.
TABLE 2  Asymmetrically expressed genes or proteins probably involved in LR determination or morphogenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Product/Role</th>
<th>Side</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM-GRASP</td>
<td>Zebrafish</td>
<td>Adhesion protein</td>
<td>Left (hepatic diverticulum)</td>
<td>Schilling et al. 1999</td>
</tr>
<tr>
<td>Fli-1</td>
<td>Zebrafish</td>
<td>Transcription factor</td>
<td>Left (cardiac mesoderm)</td>
<td>Schilling et al. 1999</td>
</tr>
<tr>
<td>HGF</td>
<td>Chick</td>
<td>Kringle signaling molecule</td>
<td>Left</td>
<td>Streit et al. 1995</td>
</tr>
<tr>
<td>hLAMP-1</td>
<td>Chick</td>
<td>Extracellular matrix component</td>
<td>Left</td>
<td>Smith et al. 1997</td>
</tr>
<tr>
<td>Rtk2</td>
<td>Zebrafish</td>
<td>Eph receptor</td>
<td>Right (foregut)</td>
<td>Schilling et al. 1999</td>
</tr>
<tr>
<td>Wnt-8C</td>
<td>Chick</td>
<td>Wnt-family member</td>
<td>Right</td>
<td>Levin 1998</td>
</tr>
<tr>
<td>XBap</td>
<td>Xenopus</td>
<td>Transcription factor</td>
<td>Left</td>
<td>Newman et al. 1997</td>
</tr>
</tbody>
</table>

*aProtein epitope.

or secretion, etc. It has been exceedingly difficult to provide experimental confirmation for this or any other theoretical model, but as discussed below, aspects are likely to be true, and numerous experiments have put important constraints on the general scheme.

Once the orientation of LR asymmetry is determined relative to the AP and DV body axes, a cascade of asymmetric gene expression reinforces the polarity and eventually provides laterality cues to the tissues that form the heart and other viscera. The characterization of these genes and their protein products permits molecular asymmetry to be detected well before morphological asymmetries are apparent. Examples of commonly used markers are Sonic hedgehog on the left side of Hensen’s node and Nodal (also known as cNR-1) in the left lateral plate mesoderm (LPM), as shown in Figure 2B. The existence of unilateral patterns of gene expression raises a crucial question: How do fairly large fields of cells learn on which side of the midline they lie? It is clear that the process must differ from that used in the first step (such as the F-molecule model) to orient left and right relative to the AP and DV axes because that process only yields information about a cell in space but not relative to the midline. Thus, in the second or propagation and reinforcement phase of LR asymmetry, cascades of gene expression convey information about a cell’s location, relative to the midline, within the broad context of the whole embryo (Figure 2). Finally, in the third phase, the LR information must be transferred to the organ primordia to initiate organotypic morphogenetic programs.

The existence of genetic and teratological phenotypes that result in a loss of asymmetry (isomerism), as well those that yield reversals and randomization of asymmetry, suggests that the initial generation of asymmetry and its orientation are
TABLE 3  Non-asymmetrically expressed genes involved in LR determination

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Product/Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin</td>
<td>Xenopus</td>
<td>Tight junction protein</td>
<td>Brizuela et al. 2001</td>
</tr>
<tr>
<td>Connexins</td>
<td>Xenopus, chick</td>
<td>Gap-junction protein</td>
<td>Levin &amp; Mercola 1998c, 1999</td>
</tr>
<tr>
<td>Furin</td>
<td>Mouse</td>
<td>Serine protease</td>
<td>Constam &amp; Robertson 2000</td>
</tr>
<tr>
<td>Hfh-4</td>
<td>Mouse</td>
<td>Winged-helix transcription factor</td>
<td>Chen et al. 1998</td>
</tr>
<tr>
<td>Inv</td>
<td>Mouse</td>
<td>Unknown</td>
<td>Mochizuki et al. 1998, Yokoyama et al. 1993</td>
</tr>
<tr>
<td>KIF-3a, 3b</td>
<td>Mouse</td>
<td>Component of ciliary motor</td>
<td>Marszalek et al. 1999, Nonaka et al. 1998, Takeda et al. 1999</td>
</tr>
<tr>
<td>No turning</td>
<td>Mouse</td>
<td>Midline patterning</td>
<td>Melloy et al. 1998</td>
</tr>
<tr>
<td>Shh</td>
<td>Mouse</td>
<td>Sonic hedgehog</td>
<td>Meyers &amp; Martin 1999</td>
</tr>
<tr>
<td>SIL</td>
<td>Mouse</td>
<td>Midline patterning</td>
<td>Izraeli et al. 1999</td>
</tr>
</tbody>
</table>

separable processes. Although many models can be proposed for how asymmetry can arise (such as that observed in prokaryotic cell division and in lateral inhibition mediated by Notch and its ligands), it is difficult to propose a model for how consistent LR orientation is achieved. Thus, arguably, the most interesting aspect of LR asymmetry is step 1: in the absence of any feature of the macroscopic world that distinguishes left from right, how is this axis reliably oriented with respect to DV and AP patterning events?

Nodal Monocilia Model

The observation that human Kartagener’s syndrome patients exhibited heterotaxia and had ultrastructural defects in the dynein component of cilia (Afzelius 1976, 1985) was of great interest because it potentially provided a way to bootstrap asymmetry from molecular chirality of some ciliary component. This idea was supported by the finding that the murine iv mutation, which unbiases laterality (Lowe et al. 1996, Schreiner et al. 1993, Singh et al. 1991), encodes a putative axonemal dynein called left-right dynein (LRD) that is expressed in cells of the mouse node (Supp et al. 1997). Axonemal dynein is a component of the motor that drives ciliary motion; the chirality of this motion is intrinsic to the protein components.
Elegant experiments have furthered the idea that the intrinsic chirality of ciliary structure is the basis for LR orientation (Marszalek et al. 1999, Nonaka et al. 1998, Okada et al. 1999, Takeda et al. 1999). Knockouts of KIF3-A or KIF3-B, two microtubule-dependent kinesin motor proteins, display randomization of the situs of the viscera, as well as of the sidedness of normally asymmetric genes. Moreover, the clockwise rotation of monocilia extending ventral to the node produces a net right-to-left fluid flow that is visible by imaging fluorescent beads placed in the extraembryonic space.

Although the involvement of motors in LR asymmetry seems certain, it is not clear when the motors are required or if the ciliary rotation is crucial. The most attractive and powerful aspect of the model is that it provides a way to leverage LR asymmetry from a molecular chirality. This predicts that the involvement of motors be the very first step in determining asymmetry. Yet various motor knockouts and mutations yield different patterns of asymmetric gene expression and visceral situs phenotypes (Wagner & Yost 2000). This might indicate that the chirality of molecular motors is not the initial step of LR determination, since if it were step 1, the downstream effects of monocilia disruption would all be the same. Another observation that complicates the straightforward fluid flow model is that \textit{inv} mutants, which have almost complete situs inversus (Yokoyama et al. 1993), have slow ciliary movement with slow and turbulent but nonetheless leftward transport (Okada et al. 1999). Unfortunately, the sequence of the \textit{inv} protein product (Mochizuki et al. 1998) offers no ready solution to why these mice have situs inversus without reversed nodal flow. One possibility is that timing when the putative morphogen swept by the cilia interacts with its receptor is critical and that the slow movement is sufficient to reverse the side where the receptors are activated (see Okada et al. 1999). Alternatively, \textit{inv} might act later in the process, or nodal flow per se might not be the symmetry-breaking function of the motors.

In addition, it is hard to reconcile the mouse experiments with studies in the chick that show asymmetric gene expression prior to node formation (for example, Levin 1998, Levin et al. 1995, Stern et al. 1995) and indicate that the node itself receives LR information from surrounding tissues (Levin & Mercola 1998b, 1999; Pagan-Westphal & Tabin 1998; Psychoyos & Stern 1996; Yuan et al. 1995). Although the two species might rely on different mechanisms, it is also possible that the motor proteins are required for step 1 in both species, but earlier during embryogenesis than previously expected. Such a mechanism might reflect potential non-ciliary functions of the motors, such as asymmetric cargo transport (Levin & Mercola 1998a, Tamura et al. 1999). This model is also consistent with \textit{ld} expression in pre-streak mouse embryos (Supp et al. 1997) and expression of kinesin and dynein genes at the base of the chick primitive streak (Figure 3).

The intrinsic chirality of motor proteins is ideally suited for breaking bilateral symmetry. Crucial experiments still need to be done, however, to learn whether species other than mouse rely on molecular motors (and nodal fluid flow) to break...
symmetry. Additionally, it will be important to determine if it is, indeed, ciliary movement per se or some other, possibly earlier, role of the motor proteins that is required. Regardless of whether fluid flow is step 1 in any species, the capacity to move particles directionally is a remarkable finding, and it will be interesting to learn the nature and activity of endogenously propelled determinants. Finally, it is important to determine why ciliary rotation propels particles unilaterally. Among the possibilities, node cilia might be uniformly tilted (as bristles in the *Drosophila* wing) so that the particles away from the cell surface all move in one direction. Alternatively, ciliary rotation might accelerate during a proportion of the stroke (e.g., 9 to 3 o’clock fast and 3 to 9 o’clock slow).

**Gap Junctional Communication Requirement in Chicks and Xenopus**

Levin & Mercola (1998c, 1999) hypothesized that a circumferential path of gap junctional communication (GJC), around a zone of isolation, could be the mechanism that bridges asymmetry at the level of a cell (step 1) to the embryo-wide cascades of asymmetric gene expression (step 2). We proposed that small molecule determinants are initially randomly distributed but traverse the circumferential GJC path unidirectionally, accumulating on one side of the midline, and then induce gene expression in conventional ways. The identities of such low-molecular-weight determinants remain unknown, and their path might be only a fraction of the embryo’s circumference. Nonetheless, GJC is required to correctly pattern left LPM expression of *nodal* homologues in both chicks and frogs.

In *Xenopus* embryos, injection of mRNA encoding a dominant-negative connexin protein into dorsal blastomeres or wild-type connexins into ventral blastomeres resulted in heterotaxia and randomization of *XNr-1* expression in the absence of other developmental defects (Levin & Mercola 1998c). The converse (expression of a dominant-negative connexin in ventral blastomeres or wild-type in dorsal blastomeres) did not affect LR asymmetry. These results indicate that an endogenous pattern of GJC between dorsal and lateral blastomeres must be significantly greater than across the ventral midline, consistent with the pattern of efficient lucifer yellow dye transfer seen in early cleavage stage embryos (for instance, Brizuela et al. 2001, Guthrie 1984, Olson et al. 1991, but see Landesman et al. 2000). LR asymmetry in *Xenopus* embryos is sensitive to drugs that block or open junctions between cleavage and late gastrula stages (stage 5–12), corresponding to several hours prior to specification of asymmetric *XNr-1* and heart tube looping (stage 16–20 and stage 19–22, respectively).

Similarly in chicks, differential GJC is required upstream of asymmetric *Shh* expression in the node, and one connexin, Cx43, was implicated by treatment with specific antisense oligonucleotides or blocking antibodies (Levin & Mercola 1999). Interestingly, *Cx43* mRNA is broadly expressed in the epiblast of streak-stage embryos but not in the streak itself. Thus GJC required for LR asymmetry
may propagate signals throughout the epiblast but not across an insulating zone at the streak. In support of this model, surgical incisions made along various radii emanating from the developing node abolish node asymmetry. Although consistent with the idea that the epiblast influences node asymmetry, this observation also indicates that the information does not originate from a single source but that contiguity of the blastodisc on both sides of the midline is necessary (Levin & Mercola 1999).

Despite the strong evidence that GJC is involved in LR patterning, ablation or misexpression of Cx43 in mice does not elicit true laterality defects (Ewart et al. 1997, Reaume et al. 1995). This might indicate that asymmetry determination in mice differs from that for Xenopus and chicks, but might also reflect compensation or redundancy provided by numerous connexin proteins that often exhibit overlapping patterns of expression. The generation and analysis of transgenic mice misexpressing a dominant-negative connexin construct would be necessary to test this possibility. A serine\textsuperscript{364} to proline in a putative cytoplasmic regulatory domain of human Cx43 has been reported to occur in unrelated patients with heterotaxia (Britz-Cunningham et al. 1995). This might reflect a human laterality requirement for Cx43, but such mutations were not noted in a differently selected patient sample (Gebbia et al. 1996), raising uncertainty about its prevalence in patient populations. Nonetheless, we found that a human Cx43\textsuperscript{Ser364Pro} mutant engineered by site-directed mutagenesis (Britz-Cunningham et al. 1995) does induce heterotaxia in Xenopus embryos and appears to be both a mild hypomorph and potent antimorph (Levin & Mercola 1998c).

Figure 4 depicts a model for the involvement of GJC in laterality determination in chicks, and a similar scheme applies to Xenopus. An essential feature is circumferential GJC around a zone of junctional insulation (the streak in chick and the ventral midline in Xenopus). Consideration of the model raises several important issues. First, it is necessary to identify upstream signals that orient GJC (Olson et al. 1991). It is also necessary to identify the nature of the determinants that traverse gap junctions and downstream target genes that they regulate. Finally, it is necessary to ascertain whether these determinants travel unidirectionally through the junctional path, as predicted, and what drives them to do so. We are currently pursuing the roles of unidirectional gap junctions (Robinson et al. 1993) and electrophoretic movement in this process.

**Adhesion Junction Requirements**

Adherens junctions are required for maintaining the structural integrity of epithelial cell sheets and depend on proteins such as the calcium-dependent adhesion molecule N-cadherin. Garcia-Castro et al. (2000) recently reported that N-cadherin is asymmetrically expressed in a dynamic pattern in the early chicken embryo beginning prior to node formation. Inhibiting N-cadherin function with specific antibodies randomized the direction of heart looping and altered Snail and Pitx-2
(but not lefty or nodal) expression, suggesting that N-cadherin-dependent control of adhesion or migration functions either in parallel or following Nodal signaling.

The importance of epithelial integrity is underscored by the requirement of Claudin in LR determination. Claudin is one of the integral transmembrane proteins that make up tight (or occludens) junctions, which provide the apical seal essential for the fluid impermeability properties of epithelial sheets. Overexpression of either intact Claudin or a C-terminally truncated version in *Xenopus* embryos caused heterotaxia and bilateral expression of XNr-1 (Brizuela et al. 2001). Misexpression did not lead to a decrease in GJC, as might be expected because epithelial impermeability can affect intercellular communication, but did induce a moderate increase in GJC. Clearly more work is needed to explore how cell adhesion affects LR determination; in particular, to probe the involvement of morphogenetic and physiological properties that depend on epithelial integrity.

### Left-Right Coordinator Model

How soon after fertilization are the left and right sides of the embryo differentially patterned? Twin studies in chicks and *Xenopus* revealed that there is no irreversible LR pre-pattern prior to gastrulation (Levin et al. 1997, Nascone & Mercola 1997), and the GJC and the mouse nodal flow experiments argue the same. The idea that some LR information exists at earlier times in *Xenopus* has been inferred by the ability of an active form of Vg1, a divergent TGFβ family member, to completely invert the LR axis when misexpressed on the right side (R3 blastomere) of a *Xenopus* embryo (Hyatt et al. 1996, Hyatt & Yost 1998). This suggests that Vg1 normally acts in descendents of the L3 blastomere, which contribute to the left LPM, and the model suggests a mutual antagonism with BMP on the right side of the embryo (Ramsdell & Yost 1999). Axial inversion is specific to the activated Vg1 as it cannot be mimicked by activin. Because both proteins signal through the transcription factors Smad2 and Smad3, the difference probably reflects specificity of available ligand-binding receptors. Although the data are consistent with an early LR pattern in the pre-gastrula stage *Xenopus* embryo, the precise timing remains uncertain because the persistence of the injected mRNA to later stages raises the possibility that the injected Vg1 mimics a later signal. This question is compounded by the difficulty experienced in the detection of processed, endogenous Vg1 in early *Xenopus* embryos.

### INTERMEDIATE STEPS: PROPAGATION AND REINFORCEMENT OF LEFT-RIGHT POLARITY

#### Asymmetric Cascades of Gene Expression

Once the orientation of LR asymmetry relative to the AP and DV body axes has been determined through one or more of the mechanisms discussed above, distinct left- and right-sided signaling cascades reinforce and transmit this information
to the tissues that form the asymmetric organs. Most research into the molecular basis for LR asymmetry determination has focused on the molecular genetics of these pathways (summarized in Figure 5). The tremendous amount of feedback circuitry has been both intriguing and vexing to researchers attempting to tease apart the signaling cascades. In addition, the apparent evolutionary conservation of a number of the molecular players has been offset by those instances in which different species rely on different genes and proteins to propagate LR cues. Yet another level of complexity is introduced because the orientation of LR asymmetry depends on signaling molecules, such as Wnt and BMP isoforms, that are also involved in patterning the DV, AP, or mediolateral body axes. This latter problem is part of the broader question of how signal transduction cascades elicit distinct genetic responses at different times and in different tissues of the developing embryo. With few exceptions, this issue has not been addressed systematically in the context of LR asymmetry. Consequently, our understanding of LR asymmetry will remain limited until we can explain how the embryo interprets shared signaling cascades without confusing the messages being sent.

A number of recent reviews describe the molecular genetic pathways as well as how they reinforce and propagate the orientation of LR asymmetry (Burdine & Schier 2000, Capdevila et al. 2000, Whitman & Mercola 2000, Yost 2001) and how results obtained from experimental species are related to human laterality disorders (Casey & Hackett 2000). This section, therefore, limits its scope to summarize the key conserved and divergent features of the molecular genetic pathways used by several experimental vertebrate species to orient LR asymmetry.

**TGFβ Family Members**

Key signaling cascades involved in the determination of LR asymmetry are diagrammed in Figure 5 for the chick, mouse, frog, and zebrafish. Regardless of how LR orientation is established initially in different species, the intermediate part of the determination process converges on Nodal signaling on the left side of the embryo, and recent data suggest BMP signaling on the right side of the embryo. The first evidence that Nodal might provide an essential left-sided cue came from in situ hybridization in chick, mouse, and *Xenopus*, revealing expression in the left LPM of all species and earlier expression to the left of the node in the chick (for example, see Collignon et al. 1996; Levin et al. 1995, 1997; Lohr et al. 1997; Lowe et al. 1996; Rebagliati et al. 1998; Sampath et al. 1997). Functional studies then showed that Nodal signaling activates the downstream effector Pitx2 (see below) and can alter asymmetric organ development (Levin et al. 1997, Sampath et al. 1997). Similarly, the suppression of available BMP signaling on the left side of the chicken embryo was shown to be required for Nodal expression, both adjacent to the node and in the LPM (Rodriguez Esteban et al. 1999, Yokouchi et al. 1999, Zhu et al. 1999) (discussed below). In contrast, BMP signaling on the right side of the midline suppresses the expression of Nodal.
Although ectopic activation of Nodal signaling, either by forced expression or application of Nodal protein, is sufficient to unbias downstream asymmetric gene expression and organ situs, loss-of-function experiments have only recently provided an unequivocal demonstration that Nodal is required for LR development. Because nodal is essential during gastrulation, nodal-deficient mutant mice (Conlon et al. 1991, 1994; Iannaccone et al. 1992; Zhou et al. 1993) and zebrafish (Feldman et al. 1998) arrest early, thus masking the subsequent requirement for LR asymmetric gene expression and development. Abnormal mesendoderm formation also occurs following approaches to chronically inhibit Nodal-homologue function in \textit{Xenopus} (Agius et al. 2000, Osada & Wright 1999). Confirmation of a Nodal requirement was first provided by analyses of laterality disturbances caused by mutation of \textit{cryptic} and \textit{one-eyed pinhead} (\textit{OEP}) genes (Bamford et al. 2000, J-N Chen et al. 1997, Gaio et al. 1999, Rebagliati et al. 1998, Yan et al. 1999), which encode related EGF/CFC proteins that are co-factors for Nodal signaling. When examined, these EGF-CFC mutant embryos have a loss of left LPM expression of \textit{Nodal}, \textit{lefty2}, and \textit{Pitx2}. More recently, Lowe et al. (2001) showed that mice heterozygous for a conditional hypomorphic mutant Nodal allele also have reduced nodal function and display laterality disturbances, including loss of \textit{Pitx2}, although they proceed through gastrulation.

\section*{Regulation of \textit{nodal} Gene Expression}

Expression of the \textit{nodal} gene (or homologues) in the left LPM appears to be a conserved feature of chordate development. Although the mechanism of \textit{nodal} induction in the left LPM is incompletely understood in any species, recent studies have revealed that a hierarchy of secreted proteins regulates \textit{nodal} in the chick. Shh expression on the left side of Hensen’s node in the chick induces left LPM expression of a chick homologue of Cerberus, which is known generally as Caronte, beginning at Hamburger-Hamilton (HH) stage 5 of development (Rodriguez Esteban et al. 1999, Yokouchi et al. 1999, Zhu et al. 1999). Chick Caronte inhibits BMP signaling, and ectopic expression on the right side of the developing embryo results in \textit{nodal} expression. Nodal, however, does not induce Caronte when applied to the right LPM (but does so in the right head mesoderm); thus LPM Caronte is presumed to function upstream of Nodal to suppress BMP. BMP signaling, if left unchecked, would otherwise suppress \textit{nodal} as it does to the right of the midline. This mechanism not only accounts for the induction of \textit{nodal} in the left LPM but also in the earlier, small domain to the left of the chick node. Caronte induction by Nodal in the head is interesting and might indicate independent control of head and visceral organ situs (Zhu et al. 1999).

The model that \textit{nodal} induction is blocked by BMP is consistent with the finding that expression of a dominant-negative BMP receptor on the right side of \textit{Xenopus} embryos induced \textit{XNr1} (Ramsdell & Yost 1999). Activated BMP receptor expressed on the left not only prevented \textit{XNr1} expression, as expected, but also led to aberrant bilateral and right-only expression, which would not be predicted by
the chick model. The chick model is also consistent with the bilateral expression of *nodal* seen in knockout mice lacking Smad5 (which, along with Smad1, mediates BMP signaling) (Chang et al. 2000).

Although BMP suppression of *nodal* might be conserved, upregulation by asymmetric Shh (and Caronte) might only function in the chick. However, despite symmetric Shh expression in the mouse (and other experimental species), Shh does influence *nodal*, most likely via feedback from other proteins such as Lefty homologues, which are divergent members of the TGFβ family (see below). Another important regulator of mouse *nodal* is FGF8. Although a right determinant in the chick (Figure 5), FGF8 is a left determinant in the mouse and induces *nodal* when misexpressed in the right LPM at ∼E8.0 (Meyers & Martin 1999). Accordingly, a significant proportion of FGF8-deficient mouse embryos either lack or have abnormal *nodal* as well as Pitx2 expression. Thus Shh and FGF8 appear to couple early node asymmetry to the cascades of gene expression, but by different mechanisms in chick and mouse.

The mapping of transcriptional regulatory elements revealed two binding sites for the winged helix transcription factor FAST within the *nodal* and *lefty2* genes, which can drive left-side-specific gene expression of reporter proteins in transgenic mice (Osada et al. 2000, Saijoh et al. 2000). FAST regulates transcription in response to Nodal and activin-like signals by interaction with Smad2, Smad3, and the common co-factor Smad4 (X. Chen et al. 1996, Y. P. Chen 1996, Watanabe & Whitman 1999, Yeo et al. 1999). These elements are sufficient to drive left LPM expression, indicating that Nodal positively regulates its own expression and that of the *lefty2* gene. Conservation of FAST elements in *Xenopus* and ascidian *nodal* homologues suggests that this positive feedback is evolutionarily ancient.

**Lefty Proteins and a Midline Barrier**

The discovery that Nodal can positively regulate its own gene expression highlights the enduring problem of explaining how Nodal signaling is constrained spatially. Earlier studies, predating the elucidation of promoter elements in the *nodal* gene, suggested the existence of a barrier at the dorsal midline of embryos to prevent signaling on one side of the embryo from interfering with cascades on the other. It has long been known, for instance, that twins conjoined at the level of the trunk often exhibit laterality disturbances and that the defects reside primarily in the right sibling. Spontaneous chick conjoined twins or experimentally induced *Xenopus* conjoined twins develop with a similar defect (Levin et al. 1997, Nascone & Mercola 1997). Molecular genetic analyses of these embryos, however, pointed out that the defects observed in the right sibling are not due to an intrinsic error in the orientation of the LR axis, but result from interference from signals that probably originate from the right flank of the left twin. A barrier model was proposed initially (see Danos & Yost 1995, King et al. 1998, Lohr et al. 1997, Yost 1998) to explain why interference does not occur during normal development and also to account for the severity of laterality defects seen in embryos with
midline anomalies or patterning deficits created by either genetic or microsurgical means.

Molecular level insight into this important problem was provided by the discovery of Lefty homologues. Lefty proteins are divergent TGFβ superfamily ligands (Heymer et al. 1997, Meno et al. 1996) that antagonize Nodal signaling in embryological assays (Bisgrove et al. 1999, Cheng et al. 2000, Meno et al. 1999, reviewed in Schier & Shen 2000). Humans and mice have two lefty genes each, although gene duplication probably occurred independently after the two species diverged. Thus there is not a one-to-one correspondence between the murine and human lefty genes. Only one lefty gene exists in chicks and Xenopus (the Xenopus gene is widely known as antivin). lefty genes, like nodal genes, are implicated in LR signaling because their expression is altered by mutations that affect organ situs (Dufort et al. 1998; Gaio et al. 1999; Heymer et al. 1997; Izraeli et al. 1999; King et al. 1998; Meno et al. 1996, 1998; Meyers & Martin 1999; Nonaka et al. 1998; Tsukui et al. 1999; Yan et al. 1999). Conversely, mouse embryos lacking lefty1 have bilateral expression of normally left-sided markers, including nodal (Meno et al. 1998). Given their ability to antagonize Nodal, Lefty1 in the midline is presumed to provide a barrier to prevent the unwanted propagation of Nodal signals to the right flank of the embryo, whereas Lefty2 acts as a feedback inhibitor within the left LPM.

How is the midline barrier established? In mice, lefty1 expression depends on Shh (Izraeli et al. 1999, Meyers & Martin 1999, Tsukui et al. 1999). Accordingly, Shh-/- mutant mice have laterality defects that include left pulmonary isomerism and cardiac defects reported to range from reversals to delayed and incomplete looping and malrotation (Meyers & Martin 1999, Tsukui et al. 1999). Because Shh is symmetrically expressed in the mouse, the laterality defects (and the alterations in nodal, lefty2, and Pitx2 seen in some Shh-/- animals) probably reflect defective barrier function provided by Lefty1, at least in part. Interestingly, retinoic acid also regulates lefty1 (see below) indicating a convergence of signaling at the point of barrier establishment.

Other Signaling Factors

As mentioned above, FGF8 is an important left regulator in the mouse but acts as a right regulator in the chick (Meyers & Martin 1999). fgf8 is expressed on the left LPM of mouse embryos and left-sided Nodal and Pitx2 is lost in FGF8-deficient mice. Conversely, FGF beads placed on the right stimulate nodal transcripts, placing FGF upstream of nodal in the left-side hierarchy. This is in contrast to the chick, where FGF8 is expressed in the streak and the right side of the node, where it acts to prevent inappropriate expression of the left pathway by suppressing Caronte (Rodriguez Esteban et al. 1999) and leads to the expression of the Snail-related (SnR) transcription factor (Boettger et al. 1999).

FGF isoforms, like Wnts, bind heparin sulfate proteoglycans (HSPGs). HSPGs have been implicated because of defects caused by treatment with xylopyranoside
(Yost 1990) and by mutation of the gene encoding a mouse glycosylation enzyme, \textit{Mgat-1} (Metzler et al. 1994). HSPGs can present secreted proteins or delimit their diffusion in extracellular matrix (Bernfield et al. 1999) and might function similarly in LR determination.

Retinoic acid, the active derivative of vitamin A, appears to play an important role in the regulation of asymmetric gene regulation in all experimental vertebrates examined. Excess RA, as well as vitamin A deficiency, are also well known to disturb laterality of asymmetric organs, in particular the heart, but probably as a confluence of effects on both AP and LR patterning. Recent experiments attempting to sort out the multiple effects of retinoid-dependent signaling show that RA treatment at the headfold stage induced bilateral \textit{nodal}, \textit{lefty}, and \textit{Pitx2} genes, whereas an RA antagonist can abolish expression in the LPM (Chazaud et al. 1999, Tsukui et al. 1999) but without affecting \textit{Shh} (Y.P. Chen et al. 1996, Smith et al. 1997). It is not yet clear how to reconcile these results with the less severe effects on asymmetric gene expression seen in vitamin A-deficient quails or mice lacking RALDH2, one of the enzymes involved in the formation of RA from vitamin A (Niederreither et al. 2001, Zile et al. 2000). Here it appears that retinoids do not regulate the sidedness of gene expression but might be required for maintaining adequate levels and, importantly, are crucial for cardiac looping morphogenesis and patterning the inflow tract. Why treatment with RA and RA antagonists cause a more severe effect is not yet resolved. A possible explanation is that findings with exogenous RA or RA antagonists led to overestimating the role of endogenous retinoids, possibly because of hyper activation of receptors or corepressors that bind to the promoters of the asymmetrically expressed genes.

**Developmental Timing and the Problem of Using Common Pathways to Provide LR Information**

How does the embryo keep from confounding patterning cues when different TGF\(\beta\) family members elicit different responses yet signal through common Smad proteins? One example of a differential outcome is the striking situs reversal caused by \textit{Vg1}, but not activin, misexpression in a right blastomere of \textit{Xenopus} embryos (discussed above). Because both \textit{Vg1} and activin signal through Smads 2 and 3, the explanation may lie in the particular ligand-binding, type II receptor produced by the responding cells.

Responsiveness to a particular TGF\(\beta\) ligand can vary with developmental stage. One example is that ectopically expressed activin phosphorylates Smad2 in \textit{Xenopus} before the mid-blastula transition (MBT), whereas \textit{Vg1} does so only after MBT (Faure et al. 2000). Additionally, endogenous activin-receptor signaling on the right side of Hensen’s node in the chick prior to HH stage 5 is thought to suppress \textit{Shh} and \textit{nodal} and, hence, suppresses leftness. This effect can be mimicked by application of activin protein on beads to the left side of an embryo at HH stage 4 (Levin et al. 1995). Application of Nodal later at HH stage 6–7 induces leftness, regardless of the side of the embryo to which it is applied. Although...
phosphorylation of Smads has not yet been examined under these conditions, the available evidence suggests that active Smad2 and Smad3 (both activated by Nodal and activin) suppress leftness before stage 5 but induce leftness after stage 6–7.

The critical nature of timing and context is likely to become a major focus of research not only in LR asymmetry but also in understanding how a remarkably small number of signaling pathways can elicit diverse and sometimes opposite outcomes. We need to understand how LR cues are interpreted without confounding dorsoventral patterning and vice-versa because both rely on common signaling via TGFβ, Wnt, and FGF family members. The multiple effects of timing and context are also difficult to untangle in experiments that rely on chronic overexpression or simple knockout technologies. Caution should be observed, therefore, in interpreting the effect of overexpression at one stage since persistence of the protein could have other effects later on.

**LATE STEPS: REGULATION OF ASYMMETRIC ORGAN MORPHOGENESIS**

Eventually, the cascades of gene expression described above must be translated into diverse morphogenetic processes. Internal organs acquire asymmetry as they develop initially (e.g., lung), by agenesis of a portion of a bilateral precursor (e.g., venous system) or by rotation or looping out of the midline (e.g., heart tube) (reviewed in Casey & Hackett 2000). Moreover, instances exist where defects in abdominal and thoracic organ asymmetry occur independently, and there are cases where loss of key regulatory proteins affect one or the other. For instance, thoracic but not abdominal organs are affected in lefty1−/− mice (Meno et al. 1998) and may reflect the normally anterior expression of Nodal. Moreover, distinct elements control abdominal and thoracic LPM expression of lefty1, suggesting that they may be controlled independently (Saijoh et al. 2000). Very little is known about how Nodal and other factors initiate and coordinate these morphogenetic processes. The best available insight is provided by the identification of several transcription factors that are the targets of the intermediate cascades of gene expression in the developing organ primordia.

**Pitx2 and Rieger Syndrome**

Pitx2 is a member of the bicoid class of paired homeodomain transcription factors. Pitx1 and Pitx3 are related proteins with partially overlapping expression patterns. Some overlap in function between these factors is expected because the highly conserved homeodomains share DNA-binding properties. Pitx1 and Pitx3 play prominent roles in pituitary and lens development, respectively, whereas Pitx2 is implicated in a distinct range of morphogenetic processes, including laterality. Human Pitx2 was first identified by positional cloning of the 4q25 locus, one of the loci associated with Rieger (or Axenfeld-Rieger) syndrome (Semina et al.
1996). Rieger syndrome is a rare autosomal-dominant disorder characterized by abnormalities in eye, tooth, and abdominal organ development. Severely affected individuals display defects in ventral body closure superficially reminiscent of ventral body closure deficits that accompany certain severe laterality disturbances. Defects in LR asymmetry are not characteristic of Rieger syndrome, presumably because of the presence of a normal allele.

*Pitx2* is expressed on the left LPM of chicken, mouse, *Xenopus*, and zebrafish embryos (Essner et al. 2000, Logan et al. 1998, Piedra et al. 1998, Ryan et al. 1998, Schweickert et al. 2000, St. Amand et al. 1998, Yoshioka et al. 1998). Expression continues to be left-sided in the mesodermal component of several asymmetric organs, such as the heart and gut. *Pitx2* is a direct transcriptional target of Nodal (or Nodal homologue) signal transduction acting through Smad2 (and presumably Smad3) and FAST complexes (Shiratori et al. 2001). As expected, therefore, *Pitx2* expression is inverted in most homozygous *inv/inv* embryos, as is *Nodal* and visceral organ situs. In homozygous *iv/iv* embryos, in which situs inversus incidence approaches 50%, *Pitx2*, like *Nodal*, exhibits randomized sidedness.

**Pitx2 and Left-Right Asymmetry**

Three differentially spliced *Pitx2* transcripts correspond to three different protein isoforms in the mouse (*Pitx2a*, *Pitx2b*, and *Pitx2c*), and a fourth (*Pitx2d*) has been noted in *Xenopus* (Essner et al. 2000, Faucourt et al. 2001, Schweickert et al. 2000). These differ in the amino-terminal portion of the protein but not the homeodomain. Of these, *Pitx2c* is expressed asymmetrically in the left LPM of *Xenopus* and mouse. Zebrafish, in contrast, show a more complex pattern, with *Pitx2c* left-sided in the developing gut mesoderm, and *Pitx2a* is left-sided in precardiac mesoderm. Zebrafish *Pitx2c* also exhibits left-sided expression in the developing diencephalon (Essner et al. 2000), where it might be involved in the Nodal-dependent asymmetric development of the habenular nuclei and the photoreceptive pineal gland (Concha et al. 2002). *Pitx2* isoforms are differentially regulated, and their distinct transcriptional activities are just being elucidated and appear to specifically regulate their own and *nodal*-homologue gene expression (Essner et al. 2000, Schweickert et al. 2000).

The initial misexpression studies of *Pitx2* revealed laterality defects in several vertebrates (Campione et al. 1999, Essner et al. 2000, Logan et al. 1998, Ryan et al. 1998). Mouse embryos lacking *Pitx2* are characterized grossly by failure of ventral body wall closure and arrested embryonic turning, probably caused by a thickening of the mesoderm and amnion on the left side of the embryo (Gage et al. 1999, Kitamura et al. 1999, Lin et al. 1999, Lu et al. 1999). Importantly, right pulmonary isomerism was noted such that both the left and right lungs frequently had four lobes (rather than the usual pattern of a single left and four right lobes). Although complex defects that resembled right atrial isomerism occurred, the hearts looped rightward and had normal expression of markers of asymmetric development, including *eHAND* and *dHAND*. Thus *Pitx2* in the mouse appears critical
for asymmetric lung development and embryonic turning. Its involvement in heart asymmetry, although quite evident, is less marked than might have been anticipated from the overexpression studies. Depletion of the asymmetric Pitx2c function with antisense oligonucleotides (chick and *Xenopus*) or an engrailed repressor-Pitx2c fusion protein (chick) unbiased the direction of heart looping (Yu et al. 2001, J. Dagle & D. Weeks, personal communication). More work is needed to understand the reason for the different cardiac phenotypes seen in these studies. Part of the answer might be that asymmetric morphogenesis of the heart and lung critically depends on the dosage of Pitx2c (Liu et al. 2001). The number of lung lobes is more sensitive to reduced Pitx2c than is atrial morphogenesis, indicating, perhaps not surprisingly, that the dosage thresholds for radically different morphogenesis processes differ. It is important to point out that Pitx2 affects multiple processes in early development, notably acting as a mediator of Nodal signaling in the pre- and early gastrula stage embryo (Essner et al. 2000, Faucourt et al. 2001). Thus relatively late situs defects seen under certain experimental conditions might reflect a potential background of perturbations resulting from alterations in earlier patterning.

To date, only one downstream target of Pitx2 has been identified, other than potential positive feedback on *Nodal* and Pitx2 genes. Using a chromatin-precipitation technique, Hjalt et al. (2001) identified procollagen lysyl hydroxylase (PLOD)-1 and -2 genes as potential targets. PLOD family enzymes hydroxylate lysines in collagens. Hydroxylysines provide carbohydrate attachment sites and form more stable intermolecular cross-links than do lysine residues. Interestingly, mutations in PLOD1 are associated with Ehlers-Danlos syndrome, kyphoscoliosis type (EDVI), which is characterized by ocular, skin, and muscular defects. Although it is intriguing to speculate, it is not yet clear whether decreased PLOD activity is involved in Rieger or related syndromes, or if PLOD activity mediates Nodal-dependent left-right asymmetric morphogenesis of chest and abdominal organs.

**Other Transcription Factors Downstream of Nodal**

Snail-related (SnR) and Nkx3.2 are two other transcription factors downstream of Nodal in the LPM that are likely to influence LR asymmetric development of organ primordia. *SnR*, which encodes a zinc finger protein, is expressed initially in a bilateral pattern in anterior LPM of streak-stage chick and mouse embryos (Isaac et al. 1997, Sefton et al. 1998). Shortly after the appearance of left LPM *nodal* transcripts, however, the left-sided *SnR* is extinguished in both species, suggesting suppression by Nodal. In the chick at least, treatment of embryos with specific antisense oligonucleotides depletes *SnR* mRNA and leads to bilateral *Pitx2* expression and randomization of heart situs (Isaac et al. 1997). The effect on *Pitx2* suggests that Nodal might induce transcription of *Pitx2*, not only by direct activation via activation of FAST/Smad2 complex but also by repression of SnR.

*Nkx3.2* encodes a homeodomain protein that is left-sided in the chick and right sided in the mouse (Schneider et al. 1999). *Nkx3.2* is repressed by Nodal in the
chick, but it is activated in the mouse. The function of Nkx3.2 in LR asymmetric morphogenesis has not yet been determined.

FUTURE PROSPECTS: NEUROLOGICAL ASYMMETRIES

While most molecular studies of LR patterning have focused on the asymmetry of the visceral organs, it should be kept in mind that the brain possesses its own asymmetry that has significant implications for cognition (Harnad 1977, McManus 1999). The basis of human handedness has been studied for many years, and genetic mechanisms are still controversial (McManus 1991, 1999).

Certain neurological or behavioral asymmetries do not correlate with visceral asymmetry (Kennedy et al. 1999, Tanaka et al. 1999). For example, individuals with complete situs inversus still have language lateralization and hand preference seen in 95% of normal right-handed individuals. Currently, it is not known if these asymmetries are established by a completely different mechanism than those described above for visceral organs. There is abundant evidence, however, that the Nodal pathway regulates at least some brain asymmetries. The brain exhibits symmetric expression of genes encoding EGF-CFC and FAST (Concha et al. 2002, Pogoda et al. 2000, Zhang et al. 1998) and asymmetric expression of nodal homologues and Pitx2 isoforms (Concha et al. 2002, Essner et al. 2000, Rebagliati et al. 1998, Sampath et al. 1998, Thisse & Thissee 1999). Moreover, the Nodal pathway regulates anatomical asymmetry in the habenulae and pineal complex in zebrafish (Concha et al. 2002). Whether certain asymmetries such as hand preference rely on the same pathways that regulate visceral asymmetry, however, has not been resolved. The middle ground might also be true; that is, both visceral organ and neurological asymmetries may depend on the same symmetry-breaking event (step 1), but the pathways may diverge later such that some neurological asymmetries (such as hand preference) might depend on a novel regulatory cascade. This would predict that human mutations that alter handedness and language, for instance, and those that affect visceral asymmetries, cluster on each branch after the fork, perhaps because viability is seriously compromised when the earliest steps are perturbed. Another fascinating problem is how to relate the pathways discussed in this review to asymmetry differences manifest between non-conjoined monozygotic twins. Although these twins do not exhibit the laterality defects that characterize conjoined twins, subtler features of mirror-image asymmetry (bookend or enantiomer twin pairs) have been reported such as hand preference, hair whorl direction, dentition errors, unilateral eye and ear defects, and even tumor locations (Beere et al. 1990, Carton & Rees 1987, Cidis et al. 1997, Gedda et al. 1981, Morison et al. 1994, Newman et al. 1937, Townsend & Richards 1990, Yager 1984). Most of these cases affect features of the head, adding weight to speculation that laterality is determined independently in the head and body. This model is reminiscent, at least superficially, of data indicating that distinct AP and DV patterning in the head and trunk is established by distinct organizers (for review, see Harland & Gerhart 1997). Although important differences distinguish AP and DV patterning from LR determination,
it is worth bearing in mind that aspects of head laterality determination might have arisen concomitantly with the head organizer and might be intimately associated with its function. Moreover, because most healthy, non-conjoined twins presumably result from separation of cleavage, morula, or early blastocyst stage embryos, it is plausible that some information is asymmetrically present in the very early mammalian embryo and is manifest when the cells are separated at an early stage. In contrast, asymmetry of the major body organs seems to be unspecified (or at least, plastic enough to be re-specified) at those stages and develops correctly in both monozygotic twins.

ACKNOWLEDGMENTS

The authors thank our many colleagues who provided data prior to publication. We also thank members of the laboratory for insightful discussions and comments. This work was supported by an Established Investigator Award from the American Heart Association (9740005N), and grants from the National Institutes of Health (HL63271 and HL59502) to MM and a Helen Hay Whitney postdoctoral fellowship (F-773) to ML.

Visit the Annual Reviews home page at www.AnnualReviews.org

LITERATURE CITED

Danos MC, Yost HJ. 1995. Linkage of cardiac left-right asymmetry and dorsal-anterior development in Xenopus. Development 121:1467–74
is required in visceral endoderm for normal primitive streak morphogenesis. Development 125:3015–25
Hyatt BA, Yost HJ. 1998. The left-right coordinator: the role of Vg1 in organizing left-right axis formation. Cell 93:37–46
Levin M, Mercola M. 1999. Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of Shh asymmetry in the node. Development 126:4703–14
Meno C, Saijoh Y, Fujii H, Ikeda M,


Ramsdell AF, Yost HJ. 1999. Cardiac looping and the vertebrate left-right axis: antagonism of left-sided Vg1 activity by a right-sided ALK2-dependent BMP pathway. *Development* 126:5195–205


genes are implicated in axial patterning and establishing left-right asymmetry. Dev. Biol. 199:261–72


in neural induction and its expression in Hensen's node during gastrulation. Development 121:813–24
Watanabe M, Whitman M. 1999. FAST-1 is a key maternal effector of mesoderm inducers in the early \textit{Xenopus} embryo. \textit{Development} 126:5621–34
Whitman M, Mercola M. 2000. TGF\textbeta superfamily signaling and left-right asymmetry. Science STKE web site (http://www.stke.org/cgi/content/full/OC_sigtrans;2000/48/re1)
Yost HJ. 1990. Inhibition of proteoglycan synthesis eliminates left-right asymmetry in \textit{Xenopus laevis} cardiac looping. \textit{Development} 110:865–74
Zhang J, Talbot WS, Schier AF. 1998. Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand
required during gastrulation. *Cell* 92:241–51


Figure 1  The Brown and Wolpert (1990) model for step 1. A chiral molecule (schematized by an F) tethered with respect to the other axes can orient the direction of the LR axis. A ciliary motor complex in the mouse is a candidate for fulfilling the F molecule function (see text).
Figure 2  Once the direction of asymmetry is broken, some mechanism must tell individual cells whether they lie to the left or right of the midline (A) in order to specify asymmetric expression of genes such as for *nodal* (B). This must be a distinct mechanism from that used to orient the left-right vector in space (as in Figure 1), for even if cells had identical knowledge of left versus right, they would still lack knowledge of position relative to the midline.
Figure 3  In situ hybridization showing expression (arrowheads) of three genes that encode motor proteins at the base of the early streak in chicken embryos.
Figure 4  Schematic model of how gap junction communication might influence left-right asymmetry (A). Small determinants (red dots) move through intercellular gap junction channels and become asymmetrically localized where they then can influence asymmetric gene expression (blue dots). This leads during subsequent development to asymmetric gene expression in Hensen’s node (e.g., Shh, yellow patch). Thus gap junctional communication is required upstream of the canonical left-sided gene cascade that provides cues for asymmetric development of organs such as the heart and gut. 

Connexin43 mRNA is expressed in the epiblast but not streak (white arrow) of streak stage chicken embryos (B) and blocking GJC (see text) perturbs the normally left-sided Shh expression (C), often causing bilateral expression (D).
Figure 5  Principal signaling pathways in LR determination (redrawn from Burdine & Schier 2000). The midline of each organism is indicated by a dashed line, with the left side to the reader's left. Gray shading indicates that a pathway or expression of a particular gene is suppressed (e.g., nodal on the right side of chicken embryos), whereas red and green denote suppression and induction, respectively. Dashed lines indicate speculative relationships, and Smad proteins are indicated where their involvement has been demonstrated.